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TURNOVER OF ACETYLCHOLINESTERASE IN INNERVATED AND DENERVATED RAT DIAPHRAGM

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SUMMARY

1. The acetylcholinesterase (AChE) in rat diaphragms was labelled by intravenous injection of echothiophate in order to evaluate the turnover of AChE in innervated and denervated muscle *in vivo*. Echothiophate diethylphosphorylates AChE thereby inactivating it. Labelled (diethylphosphorylated) enzyme is rapidly and quantitatively reactivated with 1-methyl-2-hydroxyiminomethylpyridinium (2-PAM), so labelled (diethylphosphorylated) AChE was conveniently measured as 2-PAM-reactivatable AChE activity.

2. In homogenates *in vitro*, label is lost spontaneously (diethylphosphorylated AChE spontaneously reactivates) with a half-time of 27 h.

3. In innervated diaphragm, labelled non-end-plate AChE is lost with a half-time of 13 h. When correction is made for the spontaneous loss of label on the basis of *in vitro* measurements, this data indicates that non-end-plate AChE turns over with a half-time of about 26 h.

4. In innervated diaphragm, labelled end-plate-specific AChE is lost more slowly than non-end-plate AChE and at a rate essentially identical to the rate of spontaneous loss of label *in vitro*.

5. The rate of loss of labelled non-end-plate AChE is essentially identical in 18 h denervated and in paired innervated diaphragms.

6. The rate of loss of labelled end-plate-specific AChE is significantly faster in 18 h denervated diaphragms than in paired innervated diaphragms.

7. On the basis of these observations, hypotheses concerning the mechanisms of the denervation-induced decreases in non-end-plate and end-plate-specific AChE are formulated and discussed.

INTRODUCTION

A number of molecular forms of acetylcholinesterase (AChE) that can be separated by velocity sedimentation are found in the rat diaphragm. The available evidence indicates that these forms belong to two distinct structural classes (Bon, Vigny & Massoulie, 1979). The 4S, 6·5S, and 10S forms appear to be globular proteins designated G_1, G_2, and G_4 that are monomers, dimers, and tetramers of the basic

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catalytic subunit. The 8·5S, 12·5S, and 16S forms appear to be asymmetric proteins designated A_4, A_8, and A_{12} that consist of one, two, or three tetrameters attached to a collagen-like tail (reviewed by Massoulie, 1980). About 26% of the AChE in the rat diaphragm is specifically associated with end-plates; the balance is distributed throughout the muscle as non-end-plate enzyme (Hall, 1973; Vigny, Koenig & Rieger, 1976; Younkin, Rosenstein, Collins & Rosenberry, 1982). Non-end-plate AChE is composed almost entirely of 4S (G_1) and 10S (G_4) forms (Hall, 1973; Vigny *et al.* 1976; Bon *et al.* 1979; Younkin *et al.* 1982), although asymmetric forms clearly comprise a small component of the non-end-plate AChE in the rat diaphragm (Sketelj & Brzin, 1980; Younkin *et al.* 1982). Non-end-plate AChE is divided evenly between the external (cell surface) and intracellular compartments of the diaphragm; the external component of non-end-plate AChE is predominantly 10S AChE whereas the intracellular component is predominantly the 4S form (Younkin *et al.* 1982). End-plate-specific AChE is predominantly external asymmetric forms (Hall, 1973; Vigny *et al.* 1976; Younkin *et al.* 1982) that appear to be associated with the basal lamina at the neuromuscular junction (Hall & Kelly, 1971; Betz & Sakmann, 1973; McMahan, Sanes & Marshall, 1978; Sketelj & Brzin, 1979). Some of the external end-plate-specific asymmetric AChE may be covalently integrated into the junctional basal lamina because part (39%) of this component cannot be extracted using conventional procedures (Younkin *et al.* 1982).

Innervation has a profound influence on the AChE in skeletal muscle. When mammalian skeletal muscle is denervated, both end-plate-specific and non-end-plate AChE decrease dramatically within several days (Guth, Albers & Brown, 1964; Guth, Brown & Watson, 1967; Drachman, 1972; Hall, 1973; Davey & Younkin, 1978; Collins & Younkin, 1982). There is good evidence that the influence of nerve on skeletal muscle AChE is mediated in part by the activity (action potentials and contraction) set up in muscle by nerve (Guth, 1969; Drachman, 1972; Rieger, Koenig & Vigny, 1980; Lømo & Slater, 1980; Rubin, Schuetze, Weill & Fischbach, 1980) and in part by trophic factor(s) delivered to muscle by nerve (Younkin, Brett, Davey & Younkin, 1978; Davey, Younkin & Younkin, 1979; Fernandez, Duell & Festoff, 1979; Fernandez, Patterson & Duell, 1980; Lentz, Addis & Chester, 1981). These signals ultimately must alter the synthesis and/or the post-translational processing of AChE, but the nature of the influence of innervation on AChE metabolism is unknown.

In this study, we used echothiophate to label AChE active sites and evaluated the *in vivo* turnover of AChE in the end-plate and non-end-plate regions of innervated and denervated rat diaphragms. To be an effective label for AChE, any labelling compound should (1) be specific for AChE; (2) be readily detected; (3) form a stable complex with AChE, and (4) have minimal effect on the metabolism of AChE. Echothiophate meets these criteria reasonably well. Echothiophate rapidly and selectively diethylphosphorylates AChE, thereby inactivating it. The diethylphosphorylated enzyme is quite stable (see below) but the inhibited enzyme is rapidly and quantitatively reactivated by 1-methyl-2-hydroxyiminomethylpyridinium (2-PAM) (see Froede & Wilson, 1971). To determine how much enzyme is labelled (diethylphosphorylated), one measures the AChE activity revealed by 2-PAM reactivation. Thus, the measurement of labelled enzyme is as specific as the AChE assay employed,

and labelled enzyme is detected relatively easily. Our analysis indicates that, at 37 °C *in vitro*, diethylphosphorylated AChE reactivates spontaneously with a half-time of 27 h. This rate of spontaneous reactivation is faster than one would like but did not prevent the diethylphosphorylated enzyme from being a useful probe for evaluating AChE turnover. Any significant effect of echothiophate on the metabolism of AChE should cause a change in total AChE activity. Total AChE activity was always measured after 2-PAM reactivation and, at the doses employed in the study described below, echothiophate had no effect on total AChE activity in either the end-plate or the non-end-plate region of the diaphragm.

Our results, some of which have been presented as an abstract (Younkin, 1981), indicate that non-end-plate AChE has a half-life of about 26 h and that end-plate-specific AChE has a half-life which is significantly longer. The loss of labelled non-end-plate AChE is identical in innervated diaphragms and diaphragms denervated for 18 h, but labelled end-plate-specific AChE is lost significantly faster in the denervated muscle.

METHODS

Experiments were performed on male Sprague–Dawley rats (Zivic–Miller Co., Bellefonte, PA, U.S.A.), 125–175 g weight, maintained on a controlled day–night cycle with free access to food and water. Denervation and echothiophate injection were carried out under ether anaesthesia. To denervate the left hemidiaphragm, an incision was made in the thorax and the left intrathoracic phrenic nerve was externalized with a glass hook and sectioned leaving about 15 mm of nerve attached to the muscle.

Labelling and dissection of end-plate and non-end-plate regions

Innervated rats, or rats in which the left hemidiaphragm had been denervated 18 h earlier were given 0·200 ml of 0·24 mM-echothiophate intravenously through the tail vein. At various times after echothiophate administration, left and right hemidiaphragms were removed, trimmed to remove the rib and central tendon, and divided into anterior, posterior, and middle segments by cutting 4 mm to either side of the point at which the phrenic nerve enters the diaphragm. The middle segments which contained the point of entry of the nerve were discarded. A 3 mm wide strip of muscle containing virtually all of the end-plates was obtained from the anterior and posterior segments by cutting 2 mm from the intramuscular nerve on the rib side and 1 mm on the central tendon side (Younkin *et al.* 1982). The remaining strips served as non-end-plate muscle. Wet weights were obtained for the pooled end-plate and non-end-plate segments from each hemidiaphragm. The end-plate and non-end-plate segments were homogenized in 4·0 ml of 10 mM-sodium phosphate buffer, pH 7, containing 1 % Triton X-100. A 0·500 ml aliquot of each homogenate was treated with 0·01 ml of 10 mM-1-methyl-2-hydroxyiminomethylpyridinium (2-PAM, Sigma) for 45 min at 37 °C to reactivate labelled enzyme. The original and 2-PAM-reactivated homogenates were diluted 1:10 in 100 mM-sodium phosphate buffer and assayed for AChE.

Extraction of globular, asymmetric and non-extractable forms of AChE

In some experiments globular, asymmetric and non-extractable forms of AChE were sequentially extracted from the end-plate region of the diaphragm and globular forms of AChE were selectively extracted from the non-end-plate region. The extraction procedure employed was similar to that described earlier (Younkin *et al.* 1982). In each stage of this extraction procedure, an homogenate is prepared and centrifuged for 30 min at $39000 \times g$. The supernatant is then removed for analysis of the extracted AChE, and the pellet is homogenized to begin the next stage of the sequential extraction procedure. Extracts are maintained at 4 °C throughout the procedure.

The first extraction was carried out using low ionic strength buffer (10 mM-sodium phosphate buffer, pH 7, containing 1 % Triton X-100). The supernatant obtained (S1) contained globular forms of AChE. In the non-end-plate region only globular forms (S1) were analysed. In the end-plate region

additional extraction was carried out sequentially. The second extraction of the end-plate region was carried out with low ionic strength buffer and yielded a supernatant (S2) which contained residual globular forms. The third extraction was carried out using high ionic strength buffer (low ionic strength buffer supplemented with 1·0 M-NaCl). This yielded a supernatant (S3) which contained asymmetric forms. The final pellet was homogenized in high ionic strength buffer to give an homogenate (H4) which contained non-extractable AChE. Each fraction was 2-PAM-reactivated as described above. Original and 2-PAM-reactivated fractions were then diluted 1:10 in 100 mM-sodium phosphate buffer and assayed for AChE.

Spontaneous loss of label in vitro

To evaluate the stability of the labelled (diethylphosphorylated) enzyme, homogenates from innervated diaphragms were inhibited with echothiophate, dialysed overnight against low ionic strength buffer at 4 °C to remove echothiophate, and the rate of reappearance of AChE activity at 37 °C was measured. Control homogenates untreated with echothiophate and processed in parallel were also evaluated and showed that AChE activity was stable during the course of measurement. The rate constant for spontaneous loss of labelled AChE under these conditions is given by:

$$-kt = \ln(AP/AP_0), \quad (1)$$

where AP is the activity of diethylphosphorylated enzyme at time t and AP_0 is the activity of diethlyphosphorylated enzyme at the beginning of measurement as determined by 2-PAM reactivation and k is the rate constant for loss of label. Expression of this relationship in terms of active enzyme yields the equation which was used to evaluate the rate constant for spontaneous loss of label *in vitro*:

$$-kt = \ln(1 - A/AP_0 + A_0/AP_0), \quad (2)$$

where A is AChE activity at time t and A_0 is AChE activity at the beginning of measurement.

Acetylcholinesterase assay

AChE was assayed using a modification of the technique described by Johnson & Russell (1975). Assays were carried out in minivials (Sarstedt) by adding 50 μl of [^{3}H]acetylcholine to 200 μl samples. The [^{3}H]acetylcholine solution consisted of [^{3}H]acetylcholine (New England Nuclear, 0·33 mCi/mg) made up to a concentration of 3×10^{-4} M in 0·1 M-sodium phosphate buffer, pH 7, and diluted 1:10 with an equimolar concentration of unlabelled acetylcholine iodide (Sigma). The final concentration of acetylcholine in the assay mixture was $6{\cdot}0 \times 10^{-5}$ M. Assays were run for 15–120 min and stopped by adding 1·0 ml of 0·25 M-glycine, 1·0 M-NaCl buffer at a pH of 2·5. 4 ml of Beckman NA liquid scintillation cocktail containing 10% isoamyl alcohol was then added and the vials were shaken to extract [^{3}H]acetate into the non-aqueous scintillation cocktail. For each assay triplicate controls containing only 0·1 M-sodium phosphate buffer, pH 7, were run to evaluate the amount of [^{3}H]acetate which was not attributable to enzymatic hydrolysis. To determine the relationship between [^{3}H]acetate present and counts per minute (c.p.m.) measured, triplicate samples were run which contained an amount of purified eel electric organ AChE which had been pre-determined to be capable of hydrolysing all of the acetylcholine present. The triplicate c.p.m. measurements obtained in each assay were averaged, corrected for non-enzymatic hydrolysis, converted to picomoles [^{3}H]acetate generated during the assay, and expressed as picomoles acetylcholine (ACh) hydrolysed per minute per milligram wet weight.

RESULTS

In this study, AChE was labelled by diethylphosphorylating the enzyme with echothiophate. To evaluate the stability of the labelled (diethylphosphorylated) enzyme, homogenates from innervated diaphragms were inhibited with echothiophate and dialysed overnight at 4 °C to remove echothiophate. The rate of reappearance of AChE activity at 37 °C was then measured and is shown in Fig. 1*A*. In Fig. 1*B* the data on reappearance of AChE have been plotted according to eqn. (2) in Methods to obtain the rate constant for loss of label. Linear regression of this plot indicates

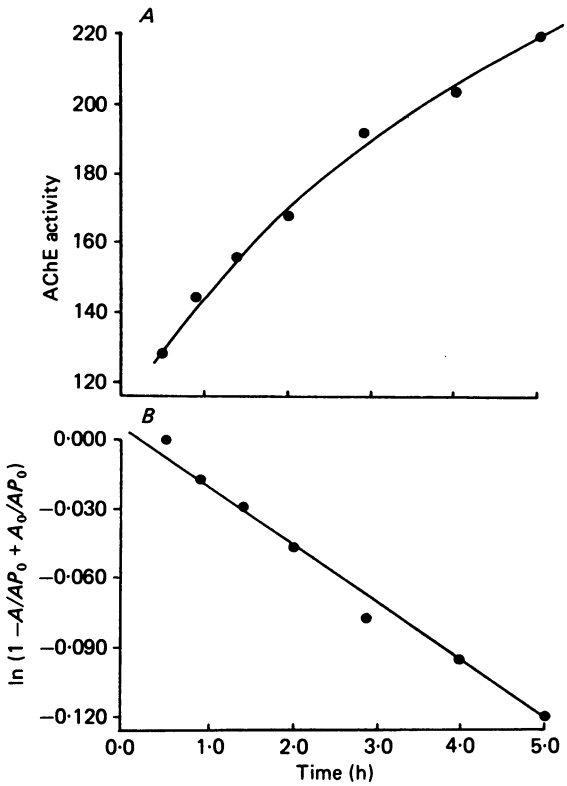

Fig. 1. Rate of reactivation of diethylphosphorylated AChE *in vitro*. In this study AChE was labelled by diethylphosphorylating the enzyme with echothiophate. Label is slowly lost through spontaneous hydrolysis which generates diethylphosphoric acid and reactivates the enzyme. To evaluate the rate at which label (diethylphosphate) is lost spontaneously, homogenates of innervated diaphragm were inactivated with echothiophate, dialysed overnight at 4 °C against low ionic strength buffer to remove echothiophate, and the recovery of enzyme activity was followed. The rate of spontaneous recovery is shown in *A*. In *B*, the data have been rearranged to fit eqn. (2) from Methods:

$$-kt = \ln(1 - A/AP_0 + A_0/AP_0),$$

where k is the rate constant for loss of label, A is AChE activity at time t, A_0 is AChE activity at the beginning of measurement, and AP_0 is the activity of diethylphosphorylated enzyme at the beginning of measurement as determined by 2-PAM reactivation. Linear regression of the data in *B* indicates that $k = 0{\cdot}026$/h. AChE activity is expressed as picomoles acetylcholine hydrolysed per minute per milligram.

that the rate constant for loss of label is 0·026/h which means that label is lost spontaneously (diethylphosphorylated AChE is spontaneously reactivated) under the conditions of this measurement with a $t_{\frac{1}{2}} = 27$ h.

AChE was labelled *in vivo* by injecting 0·200 ml of 0·24 mM-echothiophate intravenously through the tail vein. At various times after labelling, the end-plate and non-end-plate regions of the diaphragm were dissected, and the labelled enzyme in each region was determined by measuring the amount of enzyme activity that appeared when homogenates were treated with 2-PAM. The total AChE activity

measured after 2-PAM reactivation did not change as a result of echothiophate treatment in either the end-plate or the non-end-plate region of the innervated diaphragm. The total activity of the AChE in the end-plate region of the diaphragm measured after 2-PAM reactivation was 701·4 ± 16·5 pmol ACh hydrolysed/min and the total activity of the AChE in the non-end-plate region was 461·1 ± 14·2 pmol ACh hydrolysed/min . mg.

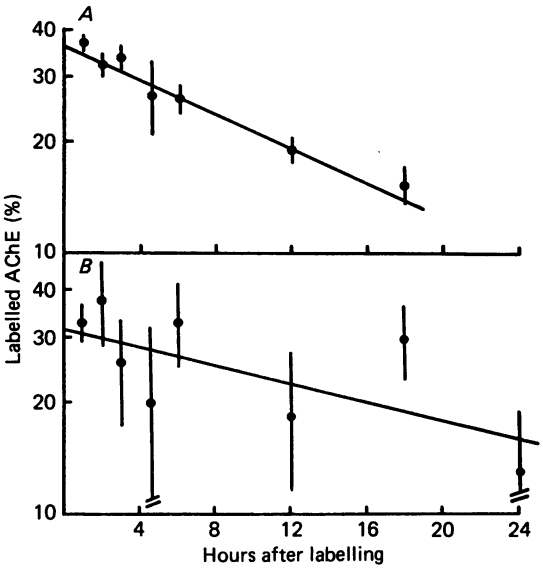

Fig. 2. Loss of labelled non-end-plate and end-plate-specific AChE. Labelled AChE is expressed as *A*, the percentage of the total non-end-plate or *B*, end-plate-specific enzyme that was reactivated by 2-PAM. Each point is the mean of five to seventeen measurements and is plotted ± S.E. of the mean.

Our protocol labelled 37 % of the AChE in the non-end-plate region. The loss of the labelled non-end-plate enzyme followed first-order kinetics and occurred at essentially the same rate in each of three separate experiments. The pooled data from all three experiments (Fig. 2*A*) indicate that labelled AChE in the non-end-plate region is lost with a half-time of 13 h. This represents a maximal rate for the turnover of non-end-plate enzyme. If one corrects for the spontaneous loss of label on the basis of the rate of reappearance of activity in homogenates, then non-end-plate enzyme is found to turn over with a half-time of 26 h.

The labelled enzyme in the end-plate region of the diaphragm consists of non-end-plate AChE as well as AChE specifically associated with the end-plate region. To evaluate the labelled enzyme specifically associated with the end-plate region, we subtracted the activity per milligram of labelled non-end-plate AChE from the total activity per milligram of the labelled enzyme found in the end-plate region. There was considerable scatter in the labelled end-plate-specific AChE measured in innervated muscle. Over-all our protocol labelled about 35 % of end-plate-specific AChE. Labelled end-plate-specific AChE was lost at about the same rate ($t_{\frac{1}{2}}$ = 30 h) that labelled enzyme was observed to reactivate spontaneously in homogenates *in vitro* ($t_{\frac{1}{2}}$ = 27 h).

We have shown previously that the external AChE in segments of rat diaphragm can be selectively inactivated *in vitro* by applying 1·25 μM-echothiophate to segments of innervated diaphragm at 0 °C for 1 h. In those experiments (Younkin *et al.* 1982), 50 % of non-end-plate AChE and 75 % of end-plate-specific AChE were inactivated and thus interpreted to be external. To selectively inactivate external AChE *in vitro*,

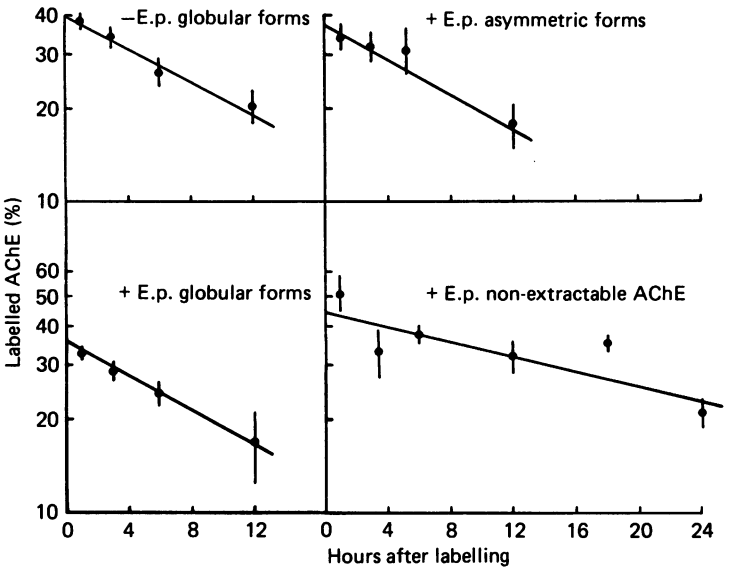

Fig. 3. Loss of labelled globular, asymmetric, and non-extractable AChE. Labelled AChE is expressed as the percentage of the total AChE in each component that was reactivated by 2-PAM. + E.p. refers to enzyme extracted from the end-plate region and − E.p. refers to enzyme extracted from the non-end-plate region. Each point is the mean of six to nine measurements and is plotted ± S.E. of the mean.

it was necessary to reduce the temperature of the preparation to 0 °C. To determine whether the enzyme labelled at 37 °C *in vivo* was external, we labelled AChE *in vivo* as described above and, 1–3 h later, removed segments of diaphragms and exposed them for 1 h to 1·25 μM-echothiophate at 0 °C for 1 h. We measured the labelled AChE in the end-plate and non-end-plate region of seventeen segments of innervated diaphragm after this procedure and found that 62 ± 1 % of the non-end-plate enzyme and 71 ± 3 % of the end-plate-specific AChE was labelled. This means that when *in vivo* labelling precedes the inactivation of external enzyme *in vitro*, an additional 12 % of non-end-plate enzyme is labelled but no additional end-plate-specific enzyme is labelled. We interpret the additional 12 % of non-end-plate AChE labelled in these control experiments to be intracellular enzyme labelled by the *in vivo* labelling procedure. This implies that the 37 % of non-end-plate enzyme labelled *in vivo* is 12 % intracellular enzyme and 25 % external enzyme. It appears, therefore, that about one-third of the 37 % of non-end-plate AChE labelled *in vivo* is intracellular whereas virtually all of the 35 % of end-plate-specific enzyme labelled *in vivo* is external.

To evaluate the turnover of particular forms of AChE, we labelled AChE intravenously with echothiophate and, at various times after labelling, extracted

globular forms from the non-end-plate region of the diaphragm and globular, asymmetric, and non-extractable forms from the end-plate region. The loss of labelled AChE in each fraction was followed as described above. The results are shown in Fig. 3. Labelled globular forms in the non-end-plate region ($t_{\frac{1}{2}}$ = 11 h), labelled globular forms in the end-plate region ($t_{\frac{1}{2}}$ = 9 h), and labelled asymmetric forms in the end-plate

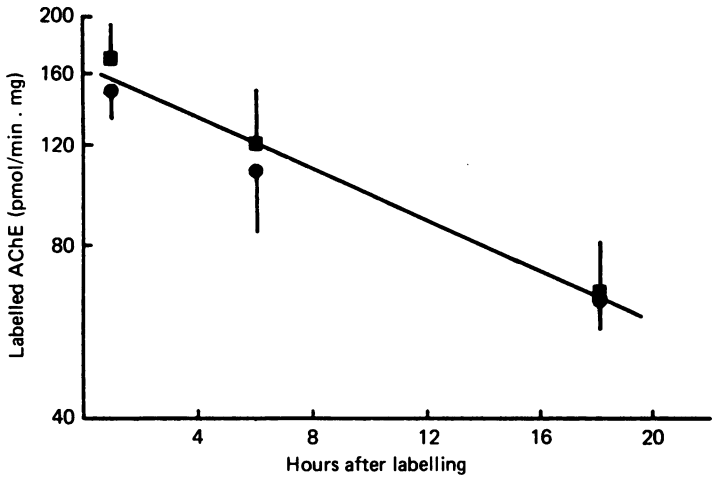

Fig. 4. Loss of labelled non-end-plate AChE in innervated and 18 h denervated rat diaphragm. The labelled AChE in innervated (■) and denervated (●) muscle is expressed as the absolute non-end-plate AChE activity (pmol ACh hydrolysed/min . mg muscle) that was reactivated by 2-PAM. Each point is the mean of four to six measurements and is plotted ±S.E. of the mean.

region ($t_{\frac{1}{2}}$ = 11 h) were all lost at essentially the same rate. The labelled non-extractable AChE in the end-plate region was lost at a slower rate ($t_{\frac{1}{2}}$ = 25 h) which was essentially identical to the rate at which label is lost from homogenates *in vitro*. It should be emphasized that forms extracted from the end-plate region are a mixture of non-end-plate enzyme and enzyme that is specifically associated with the end-plate region of the diaphragm. These fractions may not, therefore, reflect the behaviour of end-plate-specific forms.

To evaluate the effect of denervation on the turnover of AChE, we labelled AChE 18 h after denervation of the left diaphragm, a time just before the major decreases that occur first in non-end-plate and then in end-plate-specific AChE (Collins & Younkin, 1982). The turnover of the labelled AChE in denervated and contralateral innervated diaphragms was then followed during the interval when the major changes in AChE occur. The decreases in *total* end-plate-specific and non-end-plate AChE that occurred in the denervated muscle (data not shown) were essentially identical to those reported by Collins & Younkin (1982) (by 48 h non-end-plate AChE decreases to 53 % of normal and end-plate-specific AChE to 36 % of normal). The loss of *labelled* AChE in the non-end-plate region is shown in Fig. 4 where it can be seen that the loss of labelled non-end-plate AChE was essentially identical in denervated and paired innervated diaphragms ($t_{\frac{1}{2}}$ = 13 h). The loss of labelled end-plate-specific AChE is

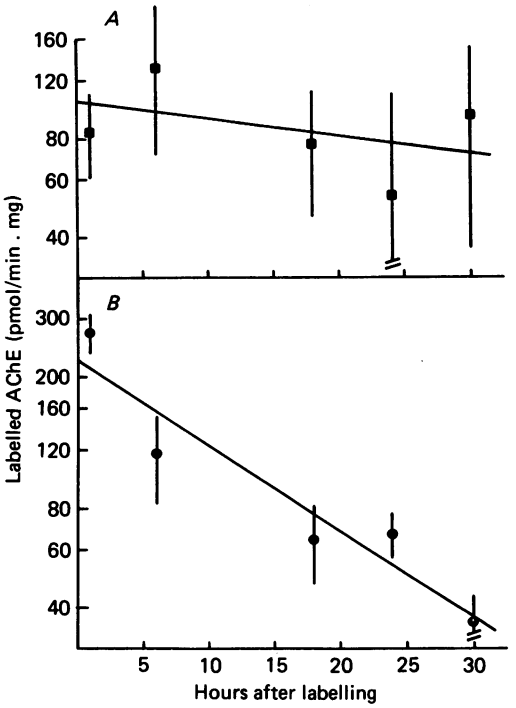

Fig. 5. Loss of labelled end-plate-specific AChE in innervated and 18 h denervated rat diaphragm. The labelled AChE in *A*, innervated and *B*, denervated muscle is expressed as the absolute end-plate-specific AChE activity (pmol ACh hydrolysed/min . mg muscle) that was reactivated by 2-PAM. Each point is the mean of two to six measurements and is plotted ±S.E. of the mean.

shown in Fig. 5 where it can be seen that the loss of labelled end-plate-specific AChE was significantly faster in denervated ($t_{\frac{1}{2}}$ = 11·5 h, Fig. 5*B*) than in paired innervated diaphragms ($t_{\frac{1}{2}}$ = 28 h, Fig. 5*A*).

DISCUSSION

Innervation has a profound influence on the AChE in the rat diaphragm. Denervation of the diaphragm causes complex changes in AChE which consist of at least three components: (1) a rapid decrease in non-end-plate AChE, (2) a large decrease in end-plate-specific AChE which begins after a delay of 1 day, and (3) a recovery phase beginning about 3 days after denervation in which both end-plate-specific and non-end-plate AChE recover but not to innervated levels (Collins & Younkin, 1982). The aim of this study was to evaluate the turnover of AChE in the early period after denervation when both non-end-plate and end-plate-specific AChE are rapidly decreasing.

Muscle cells produce three major types of AChE: asymmetric forms, soluble globular forms which are secreted, and intrinsic sarcolemmal globular forms which are externalized with their active sites exposed (for review see Massoulie, 1980; see

also Rotundo & Fambrough, 1980*a*). On the basis of evidence presented in a separate report (Younkin *et al.* 1982), we have developed a working hypothesis to account for the major features of the metabolism of AChE in the rat diaphragm. This hypothesis, which has been influenced by the studies of Rotundo & Fambrough (1980*a*, *b*) on AChE metabolism in embryonic chick myotubes, is outlined below because it provides a useful context for considering the changes in AChE metabolism caused by denervation. We propose that metabolism begins with the synthesis of the G_1 (4S) form. This form is used for the intracellular assembly of G_4 (10S) and asymmetric forms which are then externalized. The asymmetric forms which are externalized associate with the basal lamina surrounding each muscle fibre and concentrate at the neuromuscular junction, but the metabolic fate of the externalized asymmetric enzyme and the mechanism(s) responsible for concentrating this component at the neuromuscular junction are unclear. Part of the G_4 form that is externalized is secreted; the remainder stays associated with the sarcolemma as an integral membrane glycoprotein with its active sites exposed. Some G_1 AChE is also externalized as secreted and/or sarcolemmal protein but, in rat diaphragm, the G_4 form is the dominant external globular form. The metabolic fate of sarcolemmal AChE is presumably to be internalized and then rapidly degraded to amino acids in much the same way as the sarcolemmal acetylcholine receptor (Fambrough, 1979).

To evaluate the changes in AChE turnover caused by denervation, we labelled AChE with echothiophate and compared the rates of loss of labelled end-plate-specific and non-end-plate enzyme in innervated and 18 h denervated diaphragms. Our protocol labelled 35 % of the end-plate-specific AChE and 37 % of the non-end-plate enzyme in innervated muscle. From the results of control experiments, it appears that virtually all of the end-plate-specific enzyme labelled in our experimentation was external but that about one-third of the labelled non-end-plate AChE was intracellular. The labelling protocol used in this study caused no change in total AChE activity in either the end-plate or the non-end-plate region. This provides evidence that the protocol employed did not alter AChE metabolism. It was very important to keep the percentage of labelled AChE low. In preliminary experiments carried out to determine an appropriate dose of echothiophate, there was a reduction in the amount of end-plate AChE in innervated muscles when the percentage of labelled enzyme was high. We did not study this phenomenon further to determine whether high-dose echothiophate decreased synthesis or increased turnover of AChE, and it is unclear if the reduction in end-plate AChE was due to a change in the activity of the muscle or to another mechanism such as that involved in the myopathy seen after complete inactivation of AChE (Salpeter, Kasprzak, Feng & Fertuck, 1979; Leonard & Salpeter, 1979).

The rapid decrease in non-end-plate AChE in the early period after denervation (Collins & Younkin, 1982) could be due to decreased synthesis of AChE, increased secretion of AChE, accelerated degradation of intracellular AChE, or accelerated degradation of sarcolemmal AChE. Accelerated degradation of sarcolemmal AChE would accelerate the loss of labelled external non-end-plate AChE. Increased secretion and/or accelerated degradation of intracellular AChE would accelerate the loss of labelled intracellular non-end-plate AChE. In this study, we saw no change in the loss of labelled non-end-plate AChE in 18 h denervated diaphragms.

The turnover of non-end-plate AChE was measured in normal diaphragms (Fig. 2*A*), in denervated muscles (Fig. 4), and in the paired contralateral innervated muscles (Fig. 4). In each case non-end-plate AChE was found to turn over with a half-time of about 26 h after correction was made for the spontaneous loss of label on the basis of the rate of reappearance of activity in homogenates (Fig. 1). We conclude that the decrease in non-end-plate AChE in the early period after denervation is not due to accelerated degradation of sarcolemmal AChE and is unlikely to be due to increased AChE secretion or to accelerated degradation of intracellular enzyme. The evidence available indicates that the change in non-end-plate AChE after denervation is due to cessation of muscle activity (Guth, 1969; Drachman, 1972; Davey & Younkin, 1978; Fernandez *et al.* 1979; Ranish, Dettbarn & Wecker, 1980) so we propose, as a working hypothesis, that the cessation of muscle activity after denervation causes a reduction in AChE synthesis.

When the rat diaphragm is denervated, the predominantly intracellular 4S (G_1) form of AChE in the non-end-plate region decreases rapidly to a minimum of 38% of normal in 2 days, whereas the predominantly external 10S (G_4) form decreases more slowly reaching 67% of normal in 2 days and a minimum of 33% 7 days after denervation (Collins & Younkin, 1982; see also Carter & Brimijoin, 1981). A decrease in AChE synthesis would be expected to cause a rapid decrease in the first form of the enzyme that is synthesized and then a decrease in any forms that are assembled from it. Our working hypothesis is that the 4S form of the enzyme is synthesized initially and then assembled into the 10S form, so the time course of the changes in the globular forms of AChE in the non-end-plate region is consistent with the concept that denervation reduces AChE synthesis.

Labelled end-plate-specific AChE was lost from innervated diaphragms of normal rats (Fig. 2*B*, $t_{\frac{1}{2}} = 30$ h) and from contralateral (innervated) diaphragms of denervated animals (Fig. 5*A*, $t_{\frac{1}{2}} = 28$ h) at about the same rate that labelled enzyme was observed to reactivate spontaneously *in vitro* ($t_{\frac{1}{2}} = 27$ h). It appears, therefore, that end-plate-specific AChE turns over more slowly than non-end-plate enzyme, but the turnover of end-plate-specific enzyme in innervated muscle is too slow to evaluate quantitatively by following diethylphosphorylated enzyme. In 18 h denervated diaphragms, labelled end-plate-specific AChE was lost more rapidly ($t_{\frac{1}{2}} = 11{\cdot}5$ h) than in innervated muscles. The end-plate-specific AChE labelled in these experiments was external enzyme. External end-plate-specific AChE appears to be associated with the basal lamina (Hall & Kelly, 1971; Betz & Sakmann, 1973; McMahan *et al.* 1978; Sketelj & Brzin, 1979); it is predominantly (84%) asymmetric forms and about 39% of these asymmetric forms are not extracted with conventional methods (Younkin *et al.* 1982). We conclude, therefore, that denervation accelerates the degradation of external asymmetric forms of AChE that are associated with the basal lamina located between the nerve terminal and the post-synaptic muscle membrane.

End-plate-specific AChE does not decrease on the first day after denervation, but it then decreases rapidly reaching a minimum of 27% of normal 3 days after denervation (Collins & Younkin, 1982). Our analysis of the individual asymmetric forms in the end-plate region indicates that the 16S (A_{12}) form decreases very rapidly, that the 12·5S (A_8) form decreases more slowly, and that the 8·5S (A_4) form actually increases transiently as the larger forms decrease. This pattern supports the concept

that denervation accelerates the degradation of end-plate-specific asymmetric forms and suggests that this degradation causes the production of smaller asymmetric forms from the larger ones as well as the destruction of enzyme activity.

Some of the AChE specifically associated with the end-plate region of the diaphragm is undoubtedly located in the axons and terminals of the phrenic nerve. This neural AChE is presumably degraded rapidly as nerve terminals and axons degenerate after denervation, but the decrease in neural AChE is probably only a minor component of the 73 % decrease in end-plate-specific AChE seen after denervation because the quantitative data available indicate that the AChE in nerve is a minor component of total end-plate-specific AChE (Salpeter, 1967; Davey & Younkin, 1978).

Our results clearly show that accelerated degradation of end-plate-specific AChE is an important factor causing the rapid decrease in end-plate-specific enzyme that occurs 1–2 days after denervation (Fig. 5). They do not, however, establish that the *persistent* decrease in end-plate-specific AChE after denervation is due exclusively to a *persistent* increase in the turnover of end-plate-specific enzyme. Two points should be emphasized in this regard. First, the results on non-end-plate AChE suggest that denervation decreases AChE synthesis and raise the possibility that decreased synthesis might contribute to the denervation-induced decrease in end-plate-specific enzyme. Decreased synthesis could not, of course, by itself cause the rapid decrease in end-plate-specific AChE seen after denervation because end-plate-specific enzyme normally turns over too slowly, but a decreased rate of synthesis could contribute to the persistent reduction in end-plate-specific AChE that occurs. Secondly, the enzyme that is turning over rapidly in denervated diaphragm appears to be associated with the basal lamina just beneath the nerve terminal, and nerve terminal degeneration occurs after denervation at about the same time as the rapid decrease in end-plate-specific AChE (Miledi & Slater, 1970). It is possible, therefore, that the accelerated degradation of end-plate-specific AChE seen in this study does not represent a persistent increase in the turnover of end-plate-specific enzyme but is a transient event caused by increased proteolysis associated with nerve terminal degeneration. It should be possible to clarify this point by examining the turnover of end-plate-specific AChE at longer times after denervation.

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